

§Appl. No. 09/890,654
Amdt. dated November 29, 2004
Reply to Advisory Action of, October 26, 2004

REMARKS

Entry of the amendment filed October 5, 2004 is requested. The Amendment filed August 27, 2004 was not entered and should not be entered upon filing of this RCE.

In the Advisory Action dated October 26, 2004, it was stated that

“there is no page 10 of the specification, page 10 is the first page of original claims, and no mention of a standard curve is found at lines 10-15. Also, no Figure 1 is part of the specification as filed. Applicants point to the priority document of PCT/DE00/00330 to show evidence of the presence of a Figure. However, the Figure submitted in the response of 8/25/04, while part of the parent application PCT/DE00/00330, has not been translated from German to English as the rest of the specification has prior to filing in the U.S.”

It is not correct that the specification has no Page 10. Enclosed are copies of the German language application and its translation which were downloaded from the electronic file history available from the PTO website. It contains a Page 10 that refers to a standard curve. The claims begin on Page 11. These documents should be available to the examiner. If the examiner still believes that a Page 10 was not present in the translation, it is requested that the undersigned be contacted by telephone to clear this matter up expeditiously.

Figure 1 was present in the National Phase of the PCT filed in the US. The English translation of the German language application contained a Page 16 labeled “Fig. 1” and which translated the only phrase (“Hirudin concentration (µg/ml plasma”) in the figure that required it. This is adequate to satisfy the translation requirements of 37 CFR 1.495(c) and was accepted as such by the PTO. (The undersigned conferred with PCT Help Desk on this, and they agreed.) Otherwise, a notice of failure to comply would have been mailed to Applicant.

Rejection under 35 USC §112, first paragraph

The specification as filed clearly supports the amended claims. According to the

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M.P.E.P. 2163:

If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. See, e.g., *Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating “the description need not be in *ipsis verbis* [i.e., “in the same words”] to be sufficient”).

The skilled worker would have recognized that the inventors had possession, at the time the application was filed, of a kit comprising a standard curve. Reagents kits commonly are sold with standard curves and/or reagents for creating standard curves. Even when a scientist is instructed to create his or her own standard curve, an example of a curve may be provided with the kit. For examples, see Exhibit A.

The use of “standard curves” is disclosed throughout the specification, e.g., Page 3, lines 17-20; Page 4, lines 5-8; Page 9, lines 4-7; Page 10, lines 10-15; Fig. 1. Furthermore, the standard curve is expressly referred to as being “previously determined” (Page 3, lines 17-20; Page 4, lines 5-8). Consequently, the skilled worker would have understood that such a standard curve could have been included in the kit, itself.

On Page 9 of the specification, an example is provided on how to measure a compound in accordance with the methods described in the specification. The example illustrates how a standard curve is prepared, and provides guidance on how an unknown is then subsequently measured. The direction on Page 10 (“just replace the standard sample by the sample to be determined, and read the unknown hirudin concentration in Fig. 1 from the measured reduction of the optical density”) informs the skilled worker that the unknown can be measured using the same procedure, but substituting it for the known. This example does not require that the standard curve always be performed in the same experiment.

The last sentence on page 10 of the present specification text may have been translated in

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a somewhat misleading way. A better translation would be: "In an experiment the standard sample is replaced by a sample to be determined and the unknown concentration of hirudin is read out in the Figure 1 with the measured reduction of the optical density". This sentence indicates that measuring an unknown sample is done in the same way as described for measuring the standard curve. The concentration in the unknown sample is then determined by entering the measured value of the reduction of the optical density into Figure 1, the standard curve.

Additional Claim 19 has also been added that recites that the kit comprises "a known amount of hirudin for determining a standard curve." Support for Claim 19 can be found throughout the specification, e.g., Page 8, lines 5-7; and Page 9, lines 4-7.

Rejection under 35 U.S.C. §102 and §103

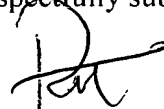
Claims 9, 10, and 17 have been amended to recite the subject matter of claims 14, 16, and 18 which were not rejected under 35 U.S.C. §102 and §103. Therefore, it is believed that the amended claims are free of the cited prior art.

In view of the above remarks, favorable reconsideration is courteously requested. If there are any remaining issues which could be expedited by a telephone conference, the Examiner is courteously invited to telephone counsel at the number indicated below.

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The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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Date: November 29, 2004

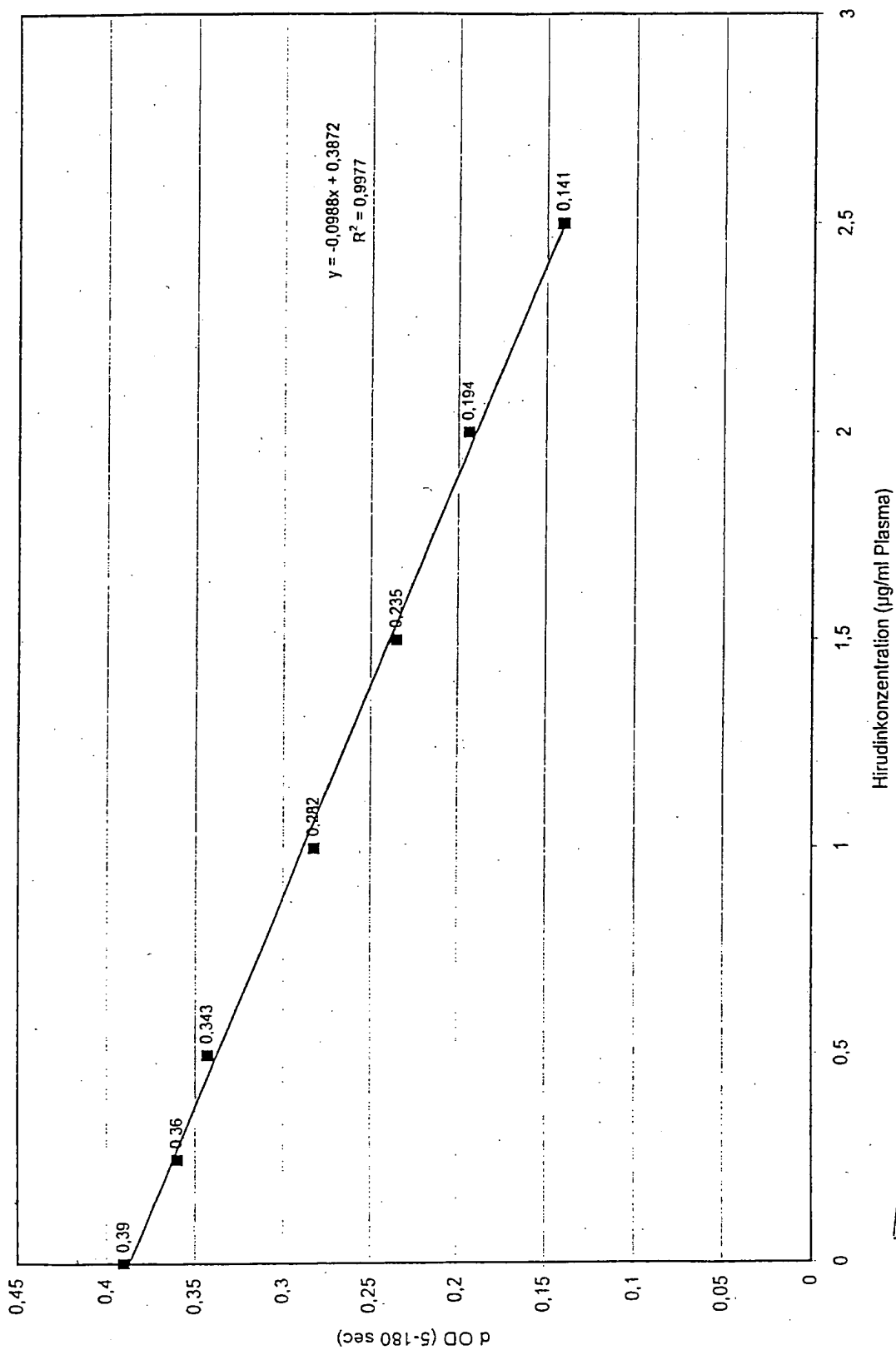


Fig. 1

EXHIBIT

A

Performance Characteristics of the Avecon **Diagnostics, Inc.** **Tetanus Antibody Test Kit**

Sensitivity -

The sensitivity of the Avecon Tetanus Antibody Test was defined as the average plus three (3) standard deviations of a series of negative serum specimens (n = 20) run with the test kit. Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.

	Target Concentration (IU/mL)	Result (IU/mL)
	0	0
	0	1
	0	1
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	1
	0	0
	0	0
	0	0
	0	1
	0	1
	0	0
	0	0
	0	1
	0	0
	0	1
	0	0
	0	1
Average		0.35
Standard		0.48

Deviation		
3 S.D.		1.4

Based on these results, it was determined that the sensitivity of the Tetanus Antibody Test is 1.8 IU/mL tetanus antibody (0.35 + 1.4).

Linearity -

1. The linearity of the Avecon Tetanus Antibody Test was determined by running successively higher concentrations of tetanus antibody added to negative serum, and determining the concentration at which the curve ceased to form a straight line. Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.

Target Concentration (IU/mL)	Result (IU/mL)
10	10
15	15
25	25
50	56
75	90
100	111
125	111
150	107

Based on these results, it was determined that the Avecon Tetanus Antibody Test is linear up to 100 IU/mL tetanus antibody.

Precision -

The precision of the Tetanus Antibody Test was determined by:

1. Assaying the quantity of tetanus antibody in the tetanus antibody reference material provided by the National Institute of Biological Standards and Controls (NIBSC, United Kingdom), Lot # 76/589. Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.
2. Determination of inter- and intra- assay precision of the test using negative serum to which was added tetanus antibody at concentrations of 10, 15 and 25 IU/mL. Tetanus antibody concentrations were quantitated using the standard curve provided

with the kit.

Results

A. NIBSC Tetanus Antibody Standard Reference Material, Lot # 76/589 (n = 6) -

	Target Concentration (IU/mL)	Result (IU/mL)
	9.2	10
	9.2	10
	9.2	9
	9.2	9
	9.2	9
	9.2	8
Mean		9.2
Standard Deviation		0.69
C.V. (%)		7.5

B. Inter-Assay Precision - Tetanus antibody was added to negative serum at three (3) different concentrations (10, 15 and 25 IU/mL) and each were run on the same day (n = 20). Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.

	10 IU/mL	15 IU/mL	25 IU/mL
	10	14	25
	10	14	25
	11	14	23
	10	15	25
	11	16	24
	10	14	23
	9	14	25
	11	15	24
	11	15	24
	10	14	25
	11	15	25

	10	15	26
	11	14	26
	10	15	24
	11	15	25
	11	15	25
	10	15	24
	11	14	24
	11	15	25
Mean	10.5	14.6	24.5
Standard Deviation	0.59	0.58	0.8
C.V. (%)	5.6	4	3.3

C. Intra-Assay Precision - Tetanus antibody was added to negative serum at three (3) different concentrations (10, 15 and 25 IU/mL) and each were assayed once per day for 15 days (n = 15). Tetanus antibody concentrations were quantitated using the standard curve provided with the kit. The same Tetanus Antibody Test kit was used for all analyses.

	10 IU/mL	15 IU/mL	25 IU/mL
	11	15	23
	11	15	25
	10	15	24
	10	15	24
	10	15	24
	10	14	25
	11	15	24
	11	15	24
	11	15	24
	11	16	24
	10	15	25
	10	15	25
	11	15	25
	11	15	24
	11	14	23
Mean	10.6	14.9	24.2

Standard Deviation	0.5	0.44	0.65
C.V. (%)	4.7	3	2.7

Interfering Substances -

The effects of interfering substances on the ability of the Tetanus Antibody Test to accurately determine the quantity of tetanus antibody in biological specimens was determined by the addition of triglycerides (750 mg/dL), hemoglobin (1000 mg/dL) and bilirubin (25 mg/dL) to a serum specimen containing 25 IU/mL tetanus antibody, and assaying the amount of tetanus antibody in the adulterated specimens using the standard curve provided with the kit. The results are as follows:

A. Triglycerides, 750 mg/dL (n = 7) -

	Target Concentration (IU/mL)	Result (IU/mL)
	25	22
	25	25
	25	28
	25	24
	25	27
	25	24
	25	26
Mean		25.1
Standard Deviation		1.9
C.V. (%)		7.6

B. Hemoglobin, 1000 mg/dL (n = 7) -

	Target Concentration (IU/mL)	Result (IU/mL)
	25	27
	25	28
	25	27
	25	28

	25	27
	25	27
	25	26
Mean		27.1
Standard Deviation		0.64
C.V. (%)		2.4

C. Bilirubin, 25 mg/dL (n = 7) -

	Target Concentration (IU/mL)	Result (IU/mL)
	25	24
	25	23
	25	24
	25	25
	25	24
	25	25
	25	25
Mean		24.3
Standard Deviation		0.7
C.V. (%)		2.9



Fluorokine[®] MAP Mouse JE/MCP-1 Kit

Catalog Number: LUM479

Pack Size: 100 Tests

Specifications and Use

Recommended Sample Types

Microparticle Region

Components

Other Supplies Required

Storage

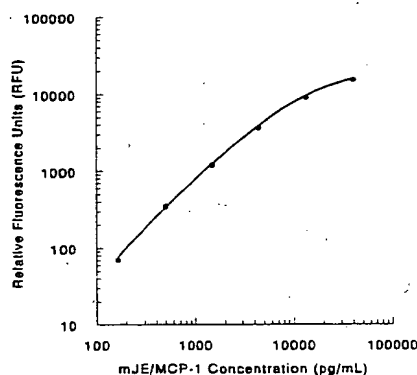
Instructions for Use

- Cell culture supernates, serum, and EDTA plasma
- Region - 30
- Microparticle Concentrate (Part 892444) is supplied as a 100X concentrated stock (0.06 mL) with preservatives.
- Biotin-Antibody Concentrate (Part 892459) is supplied as a 100X concentrated stock solution (0.06 mL) with preservatives.
- Fluorokine MAP Mouse Base Kit (Catalog Number LUM000).
- Store unopened kit at 2 - 8° C. Do not use past the expiration date on the label.
- **Avoid freezing microparticles.**
- **Protect microparticles from light.**
- Refer to the Base Kit insert for the Fluorokine MAP assay procedure.

Typical Data

This mouse JE/MCP-1 standard curve is provided only for demonstration. A standard curve must be generated each time an assay is run, utilizing values from the Standard Value Card included in the Base Kit.

Note: The Fluorokine MAP mouse JE/MCP-1 kit utilizes a six point standard curve. When fitting a standard curve constructed with the recommended 3-fold dilution series, use the first six points for the JE/MCP-1 kit only.



Standard	pg/mL	RFU	Average	Corrected
Blank	0	21 21	21	—
1	40500	15302 15285	15293	15272
2	13500	9153 8936	9044	9023
3	4500	3674 3591	3632	3611
4	1500	1208 1222	1215	1194
5	500	434 301	367	346
6	167	89 92	90	69

Performance Characteristics

All data were collected with assays run as a multiplex.

Sensitivity - The Minimum Detectable Dose (MDD) was determined by adding two standard deviations to the mean RFU of twenty zero standard replicates and calculating the corresponding concentration.

Twenty-four assays were evaluated, and the MDD of mouse JE/MCP-1 ranged from 6.2 - 37.1 pg/mL. The mean MDD was 17.5 pg/mL.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

R&D Systems, Inc.
1-800-343-7475

Intra-assay Precision (precision within an assay) - Two samples of known concentration were tested twenty times on one plate to assess precision within an assay.

Inter-assay Precision (precision between assays) - Two samples of known concentration were tested in twenty separate assays to assess precision between assays.

Intra-assay Precision Inter-assay Precision

Sample	1	2	1	2
n	20	20	20	20
Mean (pg/mL)	894	3368	996	3592
Standard Deviation	24.1	99.9	91.2	233
% CV	2.7	3.0	9.1	6.5

Recovery and Linearity - Cell culture supernate, serum, and EDTA plasma samples were spiked with natural or recombinant mouse JE/MCP-1 and evaluated for recovery and were serially diluted to evaluate assay linearity.

Recovery			Linearity				
Sample	Average % Recovery	Range (%)			Cell culture supernates	Serum	EDTA Plasma
Cell culture supernates	95	77 - 110	1:2	Average % of Expected	112	100	94
				Range (%)	92 - 122	92 - 111	93 - 95
Serum	98	74 - 117	1:4	Average % of Expected	109	98	90
				Range (%)	92 - 116	90 - 133	87 - 92
EDTA Plasma	101	97 - 104	1:8	Average % of Expected	105	97	88
				Range (%)	89 - 117	86 - 121	86 - 89

Specificity - This assay recognizes both natural and recombinant mouse JE/MCP-1. The assay was tested for interference and cross-reactivity with over 80 related factors. Less than 0.5% cross-reactivity and interference was observed.

Recombinant mouse:	LIF	Recombinant human:	IL-10	Recombinant rat:	Recombinant mouse Multiplex partners:
C10	MARC/MCP-3	GM-CSF	IL-12 p35	CNTF	GM-CSF
CTACK	M-CSF	IFN- γ	IL-12 p40	GM-CSF	IFN- γ
Eotaxin	MDC	IL-1 β	IL-12 p70	IFN- γ	IL-1 β
FAS Ligand	MIP-1 α	IL-1 R α	MCP-1	IL-1 α	IL-2
G-CSF	MIP-1 β	IL-1 sRI	MCP-2	IL-1 β	IL-4
IL-1 α	OPN	IL-1 sRII	MCP-3	IL-2	IL-5
IL-1 R α	OPG	IL-2	TNF- α	IL-4	IL-6
IL-3	OSM	IL-2 sR α	TNF sRI	IL-5	IL-10
IL-7	P/IGF-2	IL-2 sR β	TNF sRII	IL-6	IL-12 p70
IL-9	RANTES	IL-4	VEGF	IL-10	IL-13
IL-10 sR α	SCF	IL-4 sR		Leptin	KC
IL-12 p40	TECK	IL-5		TNF- α	MIP-2
IL-17	TNF sRII	IL-5 sR		VEGF ₁₆₄	TNF- α
IL-18	TPO	IL-6			VEGF
Leptin	TRANCE	IL-6 sR			

Technical Hints:

- Protect the microparticles and streptavidin-PE from light at all times.
- Refer to the Base Kit Standard Value Card for reconstitution volume and value of the reconstituted standard.
- Diluted microparticles cannot be stored. Make a fresh dilution of microparticles each time the assay is run.
- The use of a vacuum manifold device made to accommodate a microplate is necessary for washing. Adjust the vacuum to between 15 and 40 cm Hg.
- Discrepancies may exist in values obtained for the same analyte utilizing different technologies.

Fluorokine MAP affords the user the benefit of multianalyte analysis of cytokines in a complex sample. For each sample type, a single, multipurpose diluent is used to optimize recovery, linearity and reproducibility. Such a multipurpose, single diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.

ENGLISH

COATEST®

LMW Heparin / Heparin

For In Vitro Diagnostic Use

COATEST®

LMW Heparin / Heparin

Art. No. 82 13 63

CHROMOGENIX



Chromogenix -
Instrumentation Laboratory SpA
V.le Monza 338 - 20128 Milano (Italy)

302103R0

INTENDED USE OF THE KIT
For the in vitro photometric determination of the heparin or LMW heparin activity in human plasma.

MEASUREMENT PRINCIPLE

1. LMW Heparin + AT → [LMW Heparin · AT]
 - 2 a. [LMW Heparin · AT] + FXa (excess) → [LMW Heparin · AT · FXa]
 - 2 b. S-2732 + FXa → Peptide + pNA (residual)
- LMW Heparin (e.g. Fragmin) or Heparin is analyzed as a complex with Antithrombin (AT) present in the plasma sample. Activated Factor X (FXa) is added to a mixture of plasma sample and the chromogenic peptide substrate S-2732 in a buffer. Two competing reactions then start. One is the inhibition of FXa by the [LMW Heparin · AT] complex, the other is the FXa catalyzed release of pNA from the substrate. After a certain period of time most of the FXa is inhibited and the release of pNA has essentially declined. Further release of pNA is stopped by the addition of acetic acid and the absorbance at 405 nm is measured. The correlation between absorbance (A 405) and LMW Heparin/Heparin activity is linear in the 0.1–1.0 IU/ml range when plotted in a log-in scale.

REAGENTS

The sealed reagents are stable at 2-8°C until the expiration date printed on the label. Avoid contamination by microorganisms of the reagent.

1. S-2732
Chromogenic substrate (Suc-Ala-Glu-(p-*p*)-Gly-Arg-pNA), 6 mg, with mannitol as bulking agent. Reconstitute with 2.6 ml water to a concentration of 2.9 mmol/l. The solution is stable for 6 months at 2-8°C. 1 vial
2. Factor Xa
Bovine Factor Xa, 13 nkat. Reconstitute with 10.4 ml water. The solution is stable for one month at 2-8°C. 1 vial
3. Buffer, 20 ml
Tris 50 mmol/l, EDTA 7.5 mmol/l, pH 8.4, I=0.2. Once opened, the buffer solution is stable for two months at 2-8°C. 1 vial
4. LMW Heparin Standard, 1ml
A LMW Heparin (Fragmin) standard, 100 IU/ml (Calibrated against the 1st International Standard for LMW Heparin, established by the WHO, using an anti-Factor Xa method). Once opened, the solution is stable for 6 months at 2-8°C. 1 vial

Reagents required but not provided

- Deionized water, filtered through 0.22 µm or NCCLS type II water.¹
- Saline (0.9% NaCl).
- Pooled normal human plasma taken on ice and prepared according to "SPECIMEN COLLECTION". A lyophilized preparation is available from Chromogenix AB or subsidiaries.
- Stopper solution: acetic acid 20% or monosodium citrate 20%.
- Materials required but not provided
- Photometer, 405 nm
- Semi-micro cuvette (1 cm)
- Centrifuge, 2000-4000 x g
- Stopwatch

- Disposable plastic tubes
- Pipettes, calibrated

SPECIMEN COLLECTION

Nine parts of freshly drawn venous blood are collected into one part trisodium citrate. Centrifugation: 2000 x g for 10-20 minutes at 20-25°C. Refer to NCCLS document H21-A2 for further instructions on specimen collection, handling and storage.²

PROCEDURE - MANUAL TECHNIQUE

Precautions

Proper mixing is important to make sure that the reaction mixture is homogeneous, but avoid vigorous mixing as the proteins may precipitate in the foam. The method is designed for room temperature and should be kept within 32°C from the temperature used when the standard curve was established.

CALIBRATION

A standard curve is required for each new lot of the Coatest LMW Heparin/Heparin.

LMW Heparin

Add 10 µl of the LMW Heparin standard (100 IU/ml) to 1.0 ml of pooled normal plasma and mix carefully to obtain a plasma containing 1.0 IU/ml. Prepare standards by mixing this 1.0 IU/ml plasma solution with pooled normal plasma according to the table below.

Heparin

For the determination of heparin, the standard curve must be made up by using a heparin standard of known concentration (not provided). Dilute the heparin with saline in order to obtain 100 IU/ml and proceed according to the instructions for LMW Heparin above.

Plasma Standards IU/ml	Spiked plasma 1.0 IU/ml µl	Pooled normal plasma µl
0.1	25	225
0.3	75	175
0.5	125	125
0.8	200	50
1.0	250	0

These standards can be kept at -20°C or below for 6 months or at 2-8°C for two days. For routine purposes larger amounts can be produced and kept frozen in suitable aliquots.

Quality control

It is suggested that each time a test is performed one should include a LMW Heparin/Heparin plasma sample preparation, other than that used to create the standard curve. This could be a single sample obtained from any source and at a known concentration about midrange within the standard curve.

Method

Perform at room temperature (20-25°C).
S-2732 + Buffer solution
Mix 1 volume of substrate with 3 volumes of buffer. The solution is stable for at least 24 hours at room temperature or one week at 2-8°C.

Add in a test tube

Sample or standard	Plasma blank (Note 1)
S-2732 + buffer	200 µl
Buffer	400 µl
Standard or test plasma	25 µl
Factor Xa	200 µl
Mix and incubate for 8 min	-
Stopper solution (Note 2)	200 µl

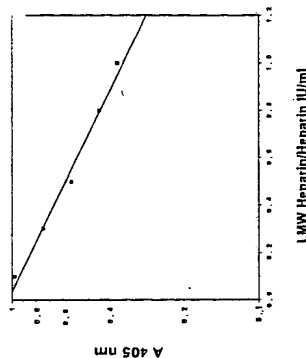
Mix

Read the absorbances at 405 nm. The colour is stable for at least 4 hours. Subtract the absorbance of the plasma blank from the sample.

Note 1. If the sample is not significantly more opaque than the standard plasma and if the bilirubin or haemoglobin content are not high enough to interfere, the pooled normal plasma blank can be used for all samples.
Note 2. The volume of the stopper solution can be chosen between 200–1000 µl in order to fill the cuvette.

CALCULATION

Plot the absorbance (A 405) for the standards (Y axis) after subtracting the plasma blank against their respective concentration of LMW Heparin/Heparin (X-axis) on loglin graph paper. Check whether A 405 for the two control samples correspond with the standard curve for the lot number of the kit. (Each laboratory must set its own guidelines for control of assay quality). Read the IU/ml value for the unknown plasma from the standard curve after subtracting the A 405 for the plasma blank.



LIMITATIONS

1. Valid determination of activities below 0.05 IU/ml may be difficult due to influence from heparin antagonists released from the platelets. Such low levels are, however, generally considered to be of limited clinical relevance.
2. If the sample contains more than 1.0 IU/ml, dilute 1:3 in pooled normal plasma and repeat the assay. Multiply the result by 3.

Expected results

To obtain an optimal effect with minimum risk of bleeding the LMW Heparin/Heparin activity should be in the range recommended by the producer. Suitable time point for sampling (also stated by the p) must be considered.

Precision

The coefficient of variation (C.V.) within serie (n=15) was 0.6% (0.8 IU/ml) and 3.3% (0.4 IU/ml). The following table shows the C.V. between series at various activities. 15 assays were performed during 4 consecutive weeks with reconstituted reagents kept at 2-8°C.

IU/ml	0.2	0.4	0.6	0.8	1.0
Heparin C.V.	6.9%	4.2%	5.7%	6.9%	8.0%
Fragmin C.V.	6.5%	5.9%	3.9%	5.7%	7.2%

ACCURACY

The assay correlates well with Coatest Heparin. Plasma samples drawn from healthy volunteers after intravenous injection of Fragmin gave a correlation coefficient of 0.98 in the activity range 0–3.5 IU/ml (n=439). In patients treated with s.c. Fragmin activities in the range 0–1.2 IU/ml gave a correlation coefficient of 0.98 (n=58).

Sensitivity

The assay allows detection of 0.05 IU/ml of LMW Heparin/Heparin. To increase the accuracy in the range below 0.1 IU/ml a 0.05 IU/ml standard is recommended.

Specificity

The assay measures specifically the anti-Factor Xa effect of LMW Heparin/Heparin. The method is slightly dependent on the patients antithrombin concentration, since antithrombin is essential for the effect of LMW Heparin/Heparin. If the result obtained deviates from the expected activity, measurement of patients antithrombin level is recommended (Coatest Antithrombin or Coacut Antithrombin available from Chromogenix or subsidiaries).



REFERENCES

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3. HA Holm et al. Heparin assays and bleeding complications in deep venous thrombosis with particular reference to retroperitoneal bleeding. *Thromb Haemostas* 53, 278-281 (1985).
4. E Holmer et al. A new simple chromogenic assay for Heparin and Heparin-like anti-FXa activity in plasma. *Thromb Haemostas* 54, 29 (1985).
5. AN Teien & M Lie. Evaluation of an amidolytic Heparin assay method: increased sensitivity by adding purified antithrombin III. *Thromb Res* 10, 399-410 (1977).
6. H ten Cate et al. Automated amidolytic method for determining Heparin, a heparinoid, and low-MR Heparin fragment, based on their anti-FXa activity. *Clin Chem* 30, 860-864 (1984).
7. JJ van Putten et al. Heparin neutralisation during collection and processing of blood inhibited by pyrodoxal 5-phosphate. *Haemostasis* 14, 253-261 (1984).
8. National Committee for Clinical Laboratory Standards. Specifications for reagent water used in the clinical laboratory, NCCLS Approved Standard: ASC-3.
9. National Committee for Clinical Laboratory Standards. Collection, transport and processing of blood specimens for coagulation testing and performance of coagulation assays. NCCLS Document H21-A2; vol. 11 No. 23

CHROMOGENIX



Enzyme Immunoassay for the Quantitative Determination of Thyroxine (T4) in Human Serum

Catalogue #KH7010

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Intended Use

For the quantitative determination of thyroxine (T4) in human serum. This kit is not licensed for use as a diagnostic test in the United States. It is therefore only sold outside the United States and is marked "for export only."

Outside the United States, the appropriateness of this test kit for research or diagnostic purposes depends on local regulations.

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Introduction

L-Thyroxine (T4) is a hormone that is synthesized and stored in the thyroid gland. Proteolytic cleavage of follicular thyroglobulin releases T4 into the bloodstream. Greater than 99% of T4 is reversibly bound to three plasma proteins in blood - thyroxine binding globulin (TBG) binds 70%, thyroxine binding pre-albumin (TBPA) binds 20%, and albumin binds 10%. Approximately 0.03% of T4 is in the free, unbound state in blood at any one time.

Diseases affecting thyroid function may present a wide array of confusing symptoms. Measurement of total T4 by immunoassay is the most reliable and convenient screening test available to determine the

presence of thyroid disorders in patients. Increased levels of T4 have been found in hyper-thyroidism due to Grave's disease and Plummer's disease and in acute and subacute thyroiditis. Low levels of T4 have been associated with congenital hypothyroidism, myxedema, chronic thyroiditis (Hashimoto's disease), and with some genetic abnormalities.

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Principle of the test

In the T-4 EIA, a certain amount of anti-T-4 antibody is coated on microtiter wells. A measured amount of patient serum, and a constant amount of T-4 conjugated with horseradish peroxidase are added to the microtiter wells. During incubation, T-4 and conjugated T-4 compete for the limited binding sites on the anti-T-4 antibody. After a 60 minutes incubation at room temperature, the wells are washed 5 times by water to remove unbound T-4 conjugate. A solution of TMB is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped and the color is changed to yellow with the addition of 2N HCl. The extent of color development is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled analyte in the sample.

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Materials

Materials provided with the test kits:

1. Antibody coated microtiter plate with 96 wells.
2. Reference standard set ready to use: 0, 1, 2.5, 5, 15, and 30.
3. Enzyme Conjugate Reagent.
4. Color Reagent A.
5. Color Reagent B.
6. 2N HCl.

Materials required but not provided:

- Precision pipettes: 50µl, 100µl, 200µl, 1ml, and 5ml.
- Disposable pipette tips.
- Distilled water.
- Glass tubes or flasks to mix Color Reagent A and Color Reagent B.
- Absorbent paper or paper towel.
- Graph paper.

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Instrumentation

The following equipment items are required to perform this assay:

- A vortex mixer or equivalent to mix reagents.
- A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-2.0 OD or greater at 450nm wavelength is required for use in the absorbance measurement.

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Storage of test kits

Unopened test kits should be stored at 2-8°C upon receipt. The microtiter plate should be stored at 2-8°C in a sealed bag with desiccants. This will minimize its exposure to damp air. Opened test kits will remain stable until the expiration date, provided they are stored as described above.

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Specimen collection and preparation

1. This kit is for use with serum samples prepared from whole blood. Blood should be drawn using standard venipuncture techniques and the serum should be separated from the red blood cells as soon as practical. Avoid grossly hemolytic, lipemic or turbid samples.
2. Plasma samples collected in tubes containing EDTA, heparin, or oxalate may interfere with the test procedures and should be avoided.
3. Specimens should be capped and may be stored up to 48 hours at 2-8°C, prior to assaying. Specimens held for a longer time can be frozen at -20°C. Thawed samples must be mixed prior to testing.

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Reagent preparation

1. All reagents should be brought to room temperature (18-25°C) and mixed by gently inverting or swirling prior to use. Do not induce foaming.
2. Add 1 ml of distilled water to reconstitute the lyophilized standards. Allow the reconstituted materials to stand for at least 20 minutes. Mix gently. The reconstituted standards should be stored sealed at 2-8 °C.
3. To prepare TMB substrate reagent, make a 1:1 dilution of Color Reagent A and Color Reagent B at least 15 minutes before use. Mix gently to ensure complete mixing. The prepared TMB substrate reagent is stable at room temperature, in the dark, for up to 3 hours. Discard excess after use.

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Assay procedures

1. Secure the desired number of coated wells in the holder.
2. Dispense 50µl of standard, specimens, and controls into appropriate wells.
3. Dispense 100µl of Enzyme Conjugate Reagent into each well.
4. Thoroughly mix for 10 seconds. It is very important to have complete mixing in this step.
5. Incubate at room temperature (18-25°C) for 60 minutes. **Prepare TMB solution 15 minutes before use.*
6. Remove the incubation mixture by flicking plate content into a waste container.
7. Rinse and flick the microtiter wells 5 times with running tap or distilled water.
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 200µl of TMB solution into each well. Gently mix for 5 seconds.
10. Incubate at room temperature for 20 minutes without shaking.
11. Stop the reaction by adding 50µl of 2N HCl to each well.
12. Gently mix for 30 seconds to make sure that the blue color changes to yellow color completely.
13. Within 30 minutes, read the optical density at 450nm with a microtiter plate reader.

Important Notes

- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
- It is recommended that no more than 32 wells be used for each assay run if manual pipetting is used since pipetting of all standards, specimens and controls should be completed within 3 minutes. A full plate of 96 well may be used if automated pipetting is available.
- Duplication of all standards and specimens, although not required is recommended.

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Calculation of results

Calculate the mean absorbance value (A_{450}) for each set of reference standards; specimens, controls and patient samples. Construct a standard curve by plotting the mean absorbance obtained from each reference standard (Y-axis) against its concentration (X-axis) on graph paper. Use the mean absorbance values for each specimen to determine the corresponding concentration of T4 from the standard curve.

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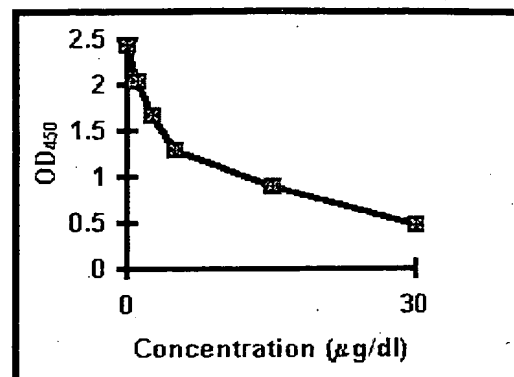
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Example of Standard Curve

Results of a typical standard run with the optical density reading at 450nm shown in the Y-axis against the T4 concentrations shown in the X-axis.

Note: This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.

T4 ($\mu\text{g/dl}$)	Absorbance (450nm)
0	2.44
1	2.05
2.5	1.66
5	1.29
15	0.89
30	0.48

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Expected values and sensitivity

T4 EIA was performed in a study of 200 euthyroid patients in one geographic location and yielded a normal of 5.0 to 13.0 $\mu\text{g/dl}$. This range corresponds to those suggested by other commercial manufacturers. It is recommended that laboratories adjust values to reflect geographic and population differences specific to the patients they serve. The minimum detectable concentration of thyroxine by this assay is estimated to be 0.4 $\mu\text{g/dl}$.

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1

Verfahren zur Bestimmung der Konzentration von Thrombininhibitoren

5 Beschreibung

Die Erfindung betrifft ein Verfahren zur Bestimmung der Konzentration von Thrombininhibitoren, wobei einem Lebewesen Körperflüssigkeit entnommen wird und wobei
10 der Körperflüssigkeit eine Prothrombin in Meizothrombin bzw. Meizothrombin-des Fragment 1 (folgend MTdesfgl) spaltende Substanz zugegeben wird. - Als Thrombininhibitoren werden alle natürlichen oder synthetische Stoffe verstanden, die Thrombin oder Thrombinvorläufer direkt inhibieren. Als Beispiel für einen
15 natürlichen Thrombininhibitor ist Hirudin zu nennen, welches aus dem Speichel von Hirudo medicinalis gewonnen werden kann. Hirudin ist ein sehr kleines Protein bestehend aus 65 Aminosäuren und mit einem Molekulargewicht von 7 kD. Beispiele für synthetische Thrombininhibitoren sind die sogenannten Hirologe, welche dem Hirudin analoge bzw. homologe Teilsequenzen aufweisen, sowie Polypeptide bestehend aus oder mit einem Tripeptid Phe-Pro-Arg oder Derivaten eines solchen Tripeptids, wie beispielsweise Borsäurederivate,
25 Chloromethylketonderivate, Benzamidinderivate, Arginine, aminosäuremodifizierte Derivate und dergleichen. Den vorstehenden Substanzen ist höchstwahrscheinlich der im wesentlichen gleiche Wirkmechanismus wie bei
30 Hirudin gemeinsam. Als Spenderlebewesen für die Körperflüssigkeit kommen Menschen und Säugetiere, wie beispielsweise Rodenten, in Frage. Beispiele für Körperflüssigkeiten sind insbesondere Blut bzw. aus Blut

hergestelltes Blutplasma. Aber auch Körperflüssigkeiten, welche kein Prothrombin enthalten, kommen in Frage, wie z.B. Urin, Liquor, Speichel, Peritonealflüssigkeit u.a. Dann wird im Rahmen der Erfindung Prothrombin zugesetzt. Nicht-trüb meint, daß keine beachtlichen Mengen an Schwabstoffen in der zu untersuchenden Körperflüssigkeit vorliegen sollen. Dies kann erforderlichenfalls beispielsweise durch Zentrifugation der Körperflüssigkeit und Abzug des Überstandes erreicht werden.

Der der Erfindung zugrundeliegende theoretische Hintergrund ist der folgende: Die Umwandlung von Prothrombin in Thrombin ist ein wesentlicher Faktor in der Blutgerinnung. Thrombin wirkt auf die Bildung von Fibrinmonomeren aus Fibrinogen sowie auf die Polymerisation der Fibrinmonomere. Prothrombin wird in Thrombin umgewandelt unter Mitwirkung von aktiviertem Faktor X, aktiviertem Faktor V Ca^{++} -Ionen und Phospholipiden, wie z.B. Plättchenfaktor 3. Hierbei findet eine mehrstufige Reaktion statt, wobei Intermediate in vergleichbar geringer Menge gebildet werden. Wenn jedoch die Koagulation mittels beispielsweise Ecarin oder einem anderen Schlangengift bzw. Schlagengiftfraktion eingeleitet wird, so entsteht demgegenüber ein "atypisches" Intermediat, beispielsweise Meizothrombin, PIVKA Meizothrombin oder Meizothrombin-des Fragment-1 (PIVKA ist eine Abkürzung für ein Protein, welches durch einen Vitamin K Antagonisten induziert wird). Diese atypischen Intermediate werden interessanterweise durch beispielsweise Hirudin inaktiviert, nicht jedoch durch Heparin (Inhibitor der Faktoren IIa, IXa, XIa, XIIa und/oder Antithrombin). Sie führen

im übrigen ebenfalls zur Thrombinbildung und subse-
quent zur Gerinnung. Die Affinität von Hirudin und
anderen synthetischen Thrombininhibitoren zu den atyp-
ischen Intermediaten ist sehr hoch ($k_i > 10^{-10}$ mol/l
5 für Meizothrombin), so daß freies atypisches Interme-
diat von dem Thrombininhibitor kurzfristig gebunden
wird.

Die vorstehenden Zusammenhänge werden in einem Ver-
10 fahren der eingangs genannten Art, welches beschrieben
ist in der Literaturstelle US-A-5,547,850, genutzt,
wobei gleichsam der Verbrauch des Thrombininhibitors
durch Messung der Verzögerung der Gerinnung erfaßt
wird. Eine große Menge an Thrombininhibitor führt zu
15 einer langen Zeit bis zum Gerinnungseintritt und um-
gekehrt. Dieses Verfahren hat sich in der Praxis
grundsätzlich ausgezeichnet bewährt. Als nachteilig
hat sich jedoch erwiesen, daß in Fällen verminderten
Fibrinogenspiegels Verfälschungen auftreten können, da
20 ein (zu) geringer Fibrinogenspiegel ebenso wie ein
hoher Thrombininhibitorspiegel zu langen Gerinnungs-
zeiten führen kann.

Der Erfindung liegt das technische Problem zugrunde,
25 ein Verfahren zur Bestimmung der Konzentration von
Thrombininhibitoren anzugeben, welches unabhängig vom
Fibrinogenspiegel genaue Werte liefert.

Zur Lösung dieses Problems lehrt die Erfindung ein
30 Verfahren zur Bestimmung der Konzentration von Throm-
bininhibitoren in einer nicht-trüben Körperflüssigkeit
oder einem nicht-trüben Extrakt aus einer Körperflüs-
sigkeit mit den folgenden Verfahrensschritten: a)

- einem Lebewesen wird die Körperflüssigkeit entnommen und die Körperflüssigkeit wird erforderlichenfalls einer Abtrennung von Trübstoffen unterworfen, b) der in Stufe a) erhaltenen nicht-trüben Körperflüssigkeit
- 5 werden ein nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw. Mtdesfgl eingreifendes gerinnungshemmendes Mittel, ein durch aktives Meizothrombin bzw. Mtdesfgl spaltbares chromogenes oder flourogenes Substrat und eine Prothrombin in Meizothrombin bzw.
- 10 Mtdesfgl-spaltende Substanz zugegeben, sowie, optional, Prothrombin, c) die in Stufe b) erhaltene Lösung bzw. Mischung wird einer wellenlängenselektiven Lichtabsorptions- oder Lichtemissionsmessung in Abhängigkeit von der Zeit unterworfen, d) aus der Ver-
- 15 minderung der Lichtabsorption oder Lichtemission in Stufe c) je Zeiteinheit wird die in der Körperflüssigkeit enthaltene Menge des Thrombininhibitors durch Vergleich mit ermittelten Standardkurven bestimmt. Alternativ zur Prothrombin in Meizothrombin bzw.
- 20 Mtdesfgl spaltenden Substanz oder ergänzend kann Meizothrombin bzw. Mtdesfgl zugegeben sein. Weiterhin lehrt die Erfindung ein Verfahren zur Bestimmung der (spezifischen) Aktivität von Thrombininhibitoren (zur Hemmung von generiertem Meizothrombin bzw. MtdesFgl)
- 25 in einer nicht-trüben wässrigen Flüssigkeit mit den folgenden Verfahrensschritten: a) einem Lebewesen wird eine Körperflüssigkeit entnommen und die Körperflüssigkeit wird erforderlichenfalls einer Abtrennung von Trübstoffen unterworfen oder eine nicht-trübe Flüssig-
- 30 keit wird künstlich hergestellt, b) der in Stufe a) erhaltenen nicht-trüben Flüssigkeit werden eine vorgegebene Menge an Thrombininhibitor, ggf. ein nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw.

Mdesfgl eingreifendes gerinnungshemmendes Mittel, ein durch aktives Meizothrombin bzw. Mdesfgl spaltbares chromogenes oder flourogenes Substrat und eine Prothrombin in Meizothrombin bzw. Mdesfgl spaltende Substanz oder Meizothrombin bzw. Mdesfgl zugegeben, sowie, optional, Prothrombin, c) die in Stufe b) erhaltene Lösung bzw. Mischung wird einer wellenlängenselektiven Lichtabsorptions- oder Lichtemissionsmessung in Abhängigkeit von der Zeit unterworfen, d) aus der Verminderung der Lichtabsorption oder Lichtemission in Stufe c) je Zeiteinheit wird die Aktivität des Thrombininhibitors durch Vergleich (des Betrages der negativen Steigung) mit ermittelten Standardkurven bestimmt. - Als chromogenes Substrat werden Substanzen bezeichnet, welche chromophore Gruppen enthalten und spezifisch von Thrombin farbgebend gespalten werden. Flourogene Substrate sind Substanzen, welche spezifisch von Thrombin unter Bildung von floureszierenden Substanzen spaltbar sind. Prothrombin kann zugesetzt werden, wenn die Körperflüssigkeit nicht natürlicherweise ausreichend Prothrombin enthält, wie beispielsweise im Falle von Vitamin K Mangel, oder wenn die zu erwartende Menge an Thrombininhibitor oder Aktivität des Thrombininhibitors dies empfiehlt, oder wenn während einer Krankheit ein Prothrombin-Mangel aufgetreten ist.

Die Erfindung beruht auf der überraschenden Erkenntnis, daß chromogene bzw. flourogene Substanzen, welche spezifisch von Thrombin gespalten werden, ebenso spezifisch von Meizothrombin bzw. Mdesfgl spaltbar sind. Dies ist nicht zu erwarten, da Intermediate zwar notwendige Vorstufen darstellen, jedoch

natürlicherweise nicht dieselbe Wirkungen bzw. Reak-
 tivitäten wie das Thrombin entfalten. Dadurch, daß die
 erfindungsgemäße Nachweisreaktion allein durch die
 Überwachung der Meizothrombin bzw. Mtdesfgl-Inhibier-
 5 ung mittels einer Farbreaktion erfolgt, ist der Nach-
 weis völlig unabhängig von dem Fibrinogenspiegel.
 Vielmehr muß beim Einsatz von Körperflüssigkeiten,
 insbesondere Blut bzw. Blutplasma, die Gerinnung sogar
 unterbunden werden, um die Farbreaktionsauswertung
 10 nicht zu stören. Zudem ist die erfindungsgemäße Throm-
 bininhibitorbestimmung in allen Bereichen mindestens
 ebenso genau, wie die Bestimmung mittels der vor-
 bekannten Methode bei hohem Fibrinogenspiegel. Auch
 besteht Unabhängigkeit von eventuell in der Körper-
 15 flüssigkeit enthaltenen, oral verabreichten Antikoagu-
 lantien. Weitere Vorteile sind: schnelle Messung
 innerhalb von Minuten in chromogenen Kanälen üblicher.
 Gerinnungsautomaten (diese messen eine Trübung oft bei
 mehreren Wellenlängen zwecks Korrektur und weisen da-
 20 her in der Regel die Möglichkeit zur wellenlängense-
 lektiven und wellenlängenvariablen Lichtabsorptions-
 messung auf); hohe Reproduzierbarkeit der gefundenen
 Werte aufgrund einer sehr niedrigen Streuung der Ein-
 zelwerte (das Konfidenzintervall liegt gemäß einer
 25 Vielzahl von Versuchsserien unter 5%, in der Regel bei
 2,2 - 3,5%); die hohe Genauigkeit bzw. Reproduzier-
 barkeit wird zudem auch bei sehr hohen Thrombininhibi-
 tor- bzw. Hirudinspiegeln erreicht; aufgrund der
 vorstehenden Eigenschaften eignet sich das erfindungs-
 30 gemäße Verfahren zur nationalen und internationalen
 Standardisierung.

Das erfindungsgemäße Verfahren findet Einsatz einerseits in der Wissenschaft, nämlich in allen Bereichen von Untersuchungen, in denen Thrombininhibitorkonzentrationen bestimmt werden müssen, sowie dem (ggf. high capacity) Screenen von prospektiven Thrombininhibitoren. In letzterem Fall kann mit hohem Durchsatz eine Vielzahl von synthetischen prospektiven Inhibitoren auf ihre tatsächliche Wirkung untersucht werden. Aktivität meint hierbei die Feststellung, ob überhaupt eine Inhibierung stattfindet und bejahendenfalls, wie die Kinetik bzw. spezifische Aktivität ist. Andererseits bietet sich auch der klinische Einsatz an, beispielsweise bei der Überwachung von Thrombininhibitorspiegeln bei Patienten, welchen der Inhibitor aus therapeutischen Gründen verabreicht wird. Auf einfache und kostengünstige Weise kann so vermieden werden, daß eine Unter- oder Überdosierung des Thrombininhibitors stattfindet, und zwar sowohl in quasi-kontinuierlicher oder diskontinuierlicher Überwachung.

Im einzelnen kann das nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw. Mdesfgl eingreifende gerinnungshemmende Mittel ausgewählt sein aus der Gruppe "Calcium-Komplexbildner, Heparin, Heparinoide, Antithrombin III, Protein C, Fibrinpolymerisationshemmstoffe und Mischungen aus diesen Stoffen". Ein konkretes Beispiel hierfür ist Pefabloc FG der Firma Pentapharm AG, Basel, Schweiz, welches ein Tetrapeptid (Gly-Pro-Arg-Pro) ist und mit hoher Affinität die Fibrinogen-Polymerisation verhindert. Die Prothrombin in Meizothrombin bzw. Mdesfgl spaltende Substanz kann ausgewählt sein aus der Gruppe der Schlangengifte und Schlangengiftfraktionen,

beispielsweise Gifte von Dispholidus, Rhabdophis, Bothrops, Notechis, Oxyuranus und Russel Viper. Zweckmäßigerweise werden gereinigte Fraktionen daraus verwendet. Vorzugsweise wird Ecarin, eine hochgradig gereinigte Fraktion des Echis-carinatus Toxins oder Multisquamase, das Prothrombin-spaltende Enzym aus Echis multisquamatus, verwendet. Solche Substanzen, wie beispielsweise Ecarin sind käuflich erwerbbar u.a. von der Firma Pentapharm AG, Schweiz.

10

Das durch aktives Meizothrombin bzw. Mtdesfgl spaltbare chromogene Substrat kann unter Spaltung p-Nitroanilin freisetzen und die Lichtabsorptionsmessung kann dann bei 405 nm durchgeführt werden. Beispiele für solche oder auch andere Substrate sind Tripeptide, welche unter den Namen Chromozym TH oder Pefachrom TH von den Firmen Chromogenix, Boehringer, Pentapharm erhältlich sind (Pefachrome TH ist H-D-ChG-Ala-Arg-pN.2AcOH). Ein Beispiel für flouochrome Substrate ist Pefachrom TH flourogen, welches unter den Namen Pefa 15865 von der Firma Pentapharm erhältlich sind.

Im einzelnen empfiehlt es sich bei den in Frage kommenden Aktivitäten, in Stufe c) eine erste Absorptions- oder Emissionsmessung nach 0 - 100 s, vorzugsweise 0 - 50, höchstvorzugsweise 5 - 15 s, und eine zweite nach anschließenden 10 - 1000 s, vorzugsweise 50 - 500 s, höchstvorzugsweise 150 - 300 s, gezählt ab Zugabe der Prothrombin in Meizothrombin bzw. Mtdesfgl spaltenden Substanz oder des Meizothrombins bzw. MTdesfgl, durchzuführen. Das erfindungsgemäße Verfahren ist insbesondere zur Bestimmung vom Hirudin oder der Bestimmung der Konzentration und/oder

der Aktivität von synthetischen Thrombininhibitoren oder Hirulogen.

- Die Erfindung betrifft weiterhin ein Test Kit zur Bestimmung der Konzentration von Thrombininhibitoren in einer nicht-trüben Körperflüssigkeit oder einem nicht-trüben Extrakt aus einer Körperflüssigkeit mit folgenden Kitkomponenten: K1) einer Lösung eines nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw.
- 10 Mtdesfgl eingreifenden gerinnungshemmenden Mittel, K2) einem durch aktives Meizothrombin bzw. Mtdesfgl spaltbaren chromogenen oder flourogenen Substrat und K3) einer Lösung einer Prothrombin in Meizothrombin bzw. Mtdesfgl spaltenden Substanz, wobei die Komponente K3)
- 15 ersetzt oder ergänzt sein kann durch eine Komponente K3a) einer Lösung mit Meizothrombin bzw. Mtdesfgl, sowie ein Test Kit zur Bestimmung der Aktivität von Thrombininhibitoren in einer nicht-trüben Körperflüssigkeit oder einem nicht-trüben Extrakt aus einer Körperflüssigkeit oder einer nicht-trüben nicht
- 20 natürlichen wäßrigen Flüssigkeit mit folgenden Kitkomponenten: optional K1) einer Lösung eines nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw. Mtdesfgl eingreifenden gerinnungshemmenden Mittel, K2)
- 25 einem durch aktives Meizothrombin bzw. Mtdesfgl spaltbaren chromogenen oder flourogenen Substrat und K3) einer Lösung einer Prothrombin in Meizothrombin bzw. Mtdesfgl spaltenden Substanz, wobei die Komponente K3) ersetzt oder ergänzt sein kann durch eine Komponente
- 30 K3a) einer Lösung mit Meizothrombin bzw. Mtdesfgl. Die Kitkomponenten können voneinander getrennt aber in einer einzigen Testkitpackung vorgesehen sein. Es kann

als optional einsetzbare zusätzliche Kitkomponente eine Lösung mit Prothrombin vorgesehen ist.

- Aufgrund des für Screeningzwecke besonders geeigneten
- 5 erfindungsgemäßen Verfahrens sind auch Gegenstand der Erfindung damit gefundene bzw. charakterisierten neue Thrombininhibitoren, welche nämlich erhältlich sind durch folgende Verfahrensschritte: A) Elemente einer Gruppe von prospektiven Thrombininhibitoren werden in
- 10 vorgebener und vorzugsweise gleicher Konzentration subsequent oder getrennt simultan einem Verfahren nach einem der Ansprüche 2 bis 8 unterworfen, B) Die Verminderung der Lichtabsorption oder Lichtemission je Zeiteinheit wird für jeden prospektiven Thrombinin-
- 15 hibitor ermittelt und mit der unter gleichen Bedingungen bestimmten Lichtabsorption oder Lichtemission je Zeiteinheit einer vorgegebenen, vorzugsweise gleichen Konzentration von Hirudin verglichen, C) es werden diejenigen prospektiven Thrombininhibitoren aus-
- 20 gewählt, deren Verminderung der Lichtabsorption oder Emission je Zeiteinheit mindestens 10% der entsprechenden Abnahme bei Einsatz von Hirudin entspricht.
- 25 Für das erfindungsgemäße Test Kit sowie die erfindungsgemäß aufgefundenen Thrombininhibitoren gelten die zum erfindungsgemäßen Verfahren getroffenen Detailerläuterungen entsprechend.
- 30 Sofern Meizothrombin bzw. Mdesfgl eingesetzt wird, so kann dies käuflich erworben werden, beispielsweise von der Fa. Pentapharm AG, Schweiz, aber auch beispielsweise gemäß der Vorschrift in der Literaturstelle

US-A-5,547,850 hergestellt werden an immobilisiertem Ecarin.

Bei den im Rahmen der Erfindung einsetzbaren Geräten
5 handelt es beispielsweise um meist ohnehin vorhandene
halb- oder vollautomatische Gerinnungsgeräte. In Frage
kommen dabei z.B. Gerinnungsautomaten des Typs Sysmex
CA-500 oder S2000 der Firma Dade-Behring oder des Typs
Electra 2000. Beim CA-500 wird das von einer LED emit-
10 tierte Licht durch einen Filter (405nm) gesandt und
anschließend durch die Probe. Das CA-500 bestimmt im
chromogenen Kanal die Änderung bzw. Verminderung der
Lichtabsorption von Farbstoffen, wie z.B. pNA (p-
Nitroanilin). Ist in einer Probe beispielsweise Hi-
15 rudin, so wird das generierte oder zugegebene Mei-
zothrombin bzw. Mdesfgl inaktiviert mit der Folge
einer dadurch behinderten pNA-Freisetzung. Die in-
sofern sich anders verhaltende (ändernde) optische
Dichte der Probe wird mittels einer Photodiode auf-
20 genommen und ausgewertet. Die zu beobachtende Lichtab-
sorptionsänderung ist umgekehrt proportional der
Hirudinaktivität.

Im folgenden wird die Erfindung anhand von lediglich
25 Ausführungsbeispiele darstellenden Experimenten näher
erläutert.

Zur Bestimmung einer Standardkurve wurde gepooltes
Humancitratplasma mit vorgegebenen Mengen Hirudin-
30 lösung versetzt. Die so erhaltenen Standardlösungen
wurden in einem CA-500 gemessen.

Als Reagenzien wurden eingefüllt,

12

Reagent 1 [Inhib] (Raumtemperatur): 400µl Pefabloc FG.
(20 mM; gelöst in 0,9% NaCl) + 2100 µl Tris-Puffer,

5 Reagent 2 [Chromol] (Raumtemperatur): Pefachrome TH
(10µmol/vial), verdünnt auf 3µmol/ml Aq. dest,

Reagent 3 [Ecarin] (15°C): Ecarin (50 EU/vial),
verdünnt auf 0,3 EU/ml (der Inhalt des Ecarinfläsch-
10 chen wird in 5ml 0,9% NaCl-Lösung gelöst und kurz vor
dem Einsatz mit einer 1:2-Mischung aus 0,9% NaCl, en-
thaltend 1% Prionex (Merck), und 0,1M CaCl₂-Lösung auf
die Endkonzentration eingestellt.

15 Das Testprotokoll ist folgend wiedergegeben. Als Dil.
Buffer wurde eingesetzt eine Mischung aus 16,6µl
Prothrombin (gereinigt; Proteingehalt: 2,22 mg/ml) und
984 µl einer Mischung aus 900µl Tris-Puffer (0,05 M,
pH 8, 37°C, + 0,1M NaCl) und 100µl Prionex (Merck).

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Testprotokoll:

Name

Ecch

Detector	Chrom
Start Point	5 sec
End Point	180 sec
Sensitivity	Low Gain
1 SampleVol.	Zitratplasma 5 µl
Dil. Vol.	Buffer 70 µl
2 SampleVol.	0 µl
.....	0 µl
Reagent 1	30 sec
Reag. Vol.	Inhib 125 µl
Rinse	125 µl
Reagent 2	120 sec
Chromo	Chromo 20 µl
Rinse	100 µl
Reagent 3	210 sec
Reag. Vol.	Ecarin 20 µl
Rinse	50 µl

(Rinse: 1%ige Natrium-Hypochloritlösung)

- 20 In der Fig. 1 ist die erhaltene Standardkurve wieder-
 gegeben. Es fällt der extrem gute Korrelationskoeff-
 fizient von 0,9977 auf. Im Experiment wird lediglich
 die Standardprobe durch eine zu bestimmende Probe er-
 setzt und die unbekannte Hirudinkonzentration in der
 25 Figur 1 anhand der gemessenen Abnahme der optischen
 Dichte abgelesen.

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Patentansprüche:

1. Verfahren zur Bestimmung der Konzentration von
Thrombininhibitoren in einer nicht-trüben Körper-
flüssigkeit oder einem nicht-trüben Extrakt aus
einer Körperflüssigkeit mit den folgenden
Verfahrensschritten:
 - a) einem Lebewesen wird die Körperflüssigkeit ent-
nommen und die Körperflüssigkeit wird erforder-
lichenfalls einer Abtrennung von Feststoffen
unterworfen,
 - b) der in Stufe a) erhaltene nicht-trübe Körper-
flüssigkeit wird in die Umwandlung
Prothrombin/aktives Meizothrombin bzw. Mdesfgl
eingreifendes gerinnungshemmendes Mittel, ein
durch aktives Meizothrombin bzw. Mdesfgl
spaltbares chromogenes oder flourogenes Sub-
strat und eine Prothrombin in Meizothrombin
bzw. Mdesfgl spaltende Substanz oder Mei-
zothrombin bzw. Mdesfgl zugegeben, sowie, op-
tional, Prothrombin,
 - c) die in Stufe b) erhaltene Lösung bzw. Mischung
wird einer wellenlängenselektiven Lichtabsorp-
tions- oder Lichtemissionsmessung in Abhängig-
keit von der Zeit unterworfen,
 - d) aus der Verminderung der Lichtabsorption oder
Lichtemission in Stufe c) je Zeiteinheit wird
die in der Körperflüssigkeit enthaltene Menge

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des Thrombininhibitors durch Vergleich mit ermittelten Standardkurven bestimmt.

2. Verfahren zur Bestimmung der Aktivität von Thrombininhibitoren in einer nicht-trüben wäßrigen Flüssigkeit mit den folgenden Verfahrensschritten:
- a) einem Lebewesen wird eine Körperflüssigkeit entnommen und die Körperflüssigkeit wird erforderlichenfalls einer Abtrennung von Trübstoffen unterworfen oder eine nicht-trübe Flüssigkeit wird künstlich hergestellt,
 - b) der in Stufe a) erhaltenen nicht-trüben Flüssigkeit werden eine vorgegebene Menge an Thrombininhibitor, ggf. ein nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw. Mtdesfgl eingreifendes gerinnungshemmendes Mittel, ein durch aktives Meizothrombin bzw. Mtdesfgl spaltbares chromogenes oder fluorogenes Substrat und eine Prothrombin in Meizothrombin bzw. Mtdesfgl spaltende Substanz oder Meizothrombin bzw. Mtdesfgl zugegeben, sowie, optional, Prothrombin,
 - c) die in Stufe b) erhaltene Lösung bzw. Mischung wird einer wellenlängenselektiven Lichtabsorptions- oder Lichtemissionsmessung in Abhängigkeit von der Zeit unterworfen,
 - d) aus der Verminderung der Lichtabsorption oder Lichtemission in Stufe c) je Zeiteinheit wird die Aktivität des Thrombininhibitors durch

- Emissionsmessung nach 0 - 100 s, vorzugsweise 0 - 50, höchstvorzugsweise 5 - 15 s, und eine zweite nach anschließenden 10 - 1000 s, vorzugsweise 50 - 500 s, höchstvorzugsweise 150 - 300 s, gezählt ab
- 5 Zugabe der Prothrombin in Meizothrombin bzw. Mtdesfgl spaltenden Substanz oder des Meizothrombins bzw. Mtdesfgl, durchgeführt werden.
- 10 8. Verfahren nach einem der Ansprüche 1 bis 7, wobei der Thrombininhibitor Hirudin, ein Hirolog oder ein synthetischer Thrombininhibitor ist.
- 15 9. Test Kit zur Bestimmung der Konzentration von Thrombininhibitoren in einer nicht-trüben Körperflüssigkeit oder einem nicht-trüben Extrakt aus einer Körperflüssigkeit mit folgenden Kitkomponenten: K1) einer Lösung eines nicht in die Umwandlung
- 20 Prothrombin/aktives Meizothrombin bzw. Mtdesfgl eingreifenden gerinnungshemmenden Mittel; K2) einem durch aktives Meizothrombin bzw. Mtdesfgl spaltbaren chromogenen oder flourogenen Substrat und K3) einer Lösung einer Prothrombin in Meizothrombin
- 25 bzw. Mtdesfgl spaltenden Substanz, wobei die Komponente K3) ersetzt oder ergänzt sein kann durch eine Komponente K3a) einer Lösung mit Meizothrombin bzw. Mtdesfgl.
- 30 10. Test Kit zur Bestimmung der Aktivität von Thrombininhibitoren in einer nicht-trüben Körperflüssigkeit oder einem nicht-trüben Extrakt aus einer Körperflüssigkeit oder einer nicht-trüben nicht

natürlichen wäßrigen Flüssigkeit mit folgenden
Kitkomponenten: optional K1) einer Lösung eines
nicht in die Umwandlung Prothrombin/aktives Meizothrombin bzw. Mdesfgl eingreifenden gerinnungshemmenden Mittel, K2) einem durch aktives
5 Meizothrombin bzw. Mdesfgl spaltbaren chromogenen
oder flourogenen Substrat und K3) einer Lösung
einer Prothrombin in Meizothrombin bzw. Mdesfgl
spaltenden Substanz, wobei die Komponente K3) er-
10 setzt oder ergänzt sein kann durch eine Komponente
K3a) einer Lösung mit Meizothrombin bzw. Mdesfgl.

11. Test Kit nach Anspruch 9 oder 10, wobei die
15 Kitkomponenten voneinander getrennt aber in einer
einzigen Testkitpackung vorgesehen sind.

12. Test Kit nach einem der Ansprüche 9 bis 11, wobei
20 als optional einsetzbare zusätzliche Kitkomponente
eine Lösung mit Prothrombin vorgesehen ist.

13. Thrombininhibitoren, welche erhältlich sind durch
25 folgende Verfahrensschritte:

A) Elemente einer Gruppe von prospektiven Thrombininhibitoren werden in vorgebener und vorzugsweise gleicher Konzentration subsequest
30 oder getrennt simultan einem Verfahren nach
einem der Ansprüche 2 bis 8 unterworfen,

19

5 B) Die Verminderung der Lichtabsorption oder Lichtemission je Zeiteinheit wird für jeden prospektiven Thrombininhibitor ermittelt und mit der unter gleichen Bedingungen bestimmten Lichtabsorption oder Lichtemission je Zeiteinheit einer vorzugsweise gleichen Konzentration von Hirudin verglichen,

10 C) es werden diejenigen prospektiven Thrombininhibitoren ausgewählt, deren Abnahme der Lichtabsorption oder Emission je Zeiteinheit mindestens 10% der entsprechenden Abnahme bei Einsatz von Hirudin entspricht.

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Zusammenfassung

Die Erfindung betrifft ein Verfahren zur Bestimmung
5 der Konzentration von Thrombininhibitoren in einer
nicht-trüben Körperflüssigkeit oder einem nicht-trüben
Extrakt aus einer Körperflüssigkeit. Sie weist die
folgenden Verfahrensschritte auf. Einem Lebewesen wird
die Körperflüssigkeit entnommen und die Körperflüssig-
10 keit wird erforderlichenfalls einer Abtrennung von
Trübstoffen unterworfen. Der so erhaltenen nicht-
trüben Körperflüssigkeit werden ein nicht in die Um-
wandlung Prothrombin/aktives Meizothrombin bzw.
Mdesfgl eingreifendes gerinnungshemmendes Mittel, ein
15 durch aktives Meizothrombin bzw. Mdesfgl spaltbares
chromogenes oder flourogenes Substrat und eine
Prothrombin in Meizothrombin bzw. Mdesfgl spaltende
Substanz zugegeben, sowie, optional, Prothrombin. Die
so erhaltene Lösung bzw. Mischung wird einer wellen-
20 längenselektiven Lichtabsorptions- oder Lichtemis-
sionsmessung in Abhängigkeit von der Zeit unterworfen.
Aus der Abnahme der Lichtabsorption oder Lichtemission
je Zeiteinheit wird die in der Körperflüssigkeit en-
thaltene Menge des Thrombininhibitors durch Vergleich
25 mit ermittelten Standardkurven bestimmt.

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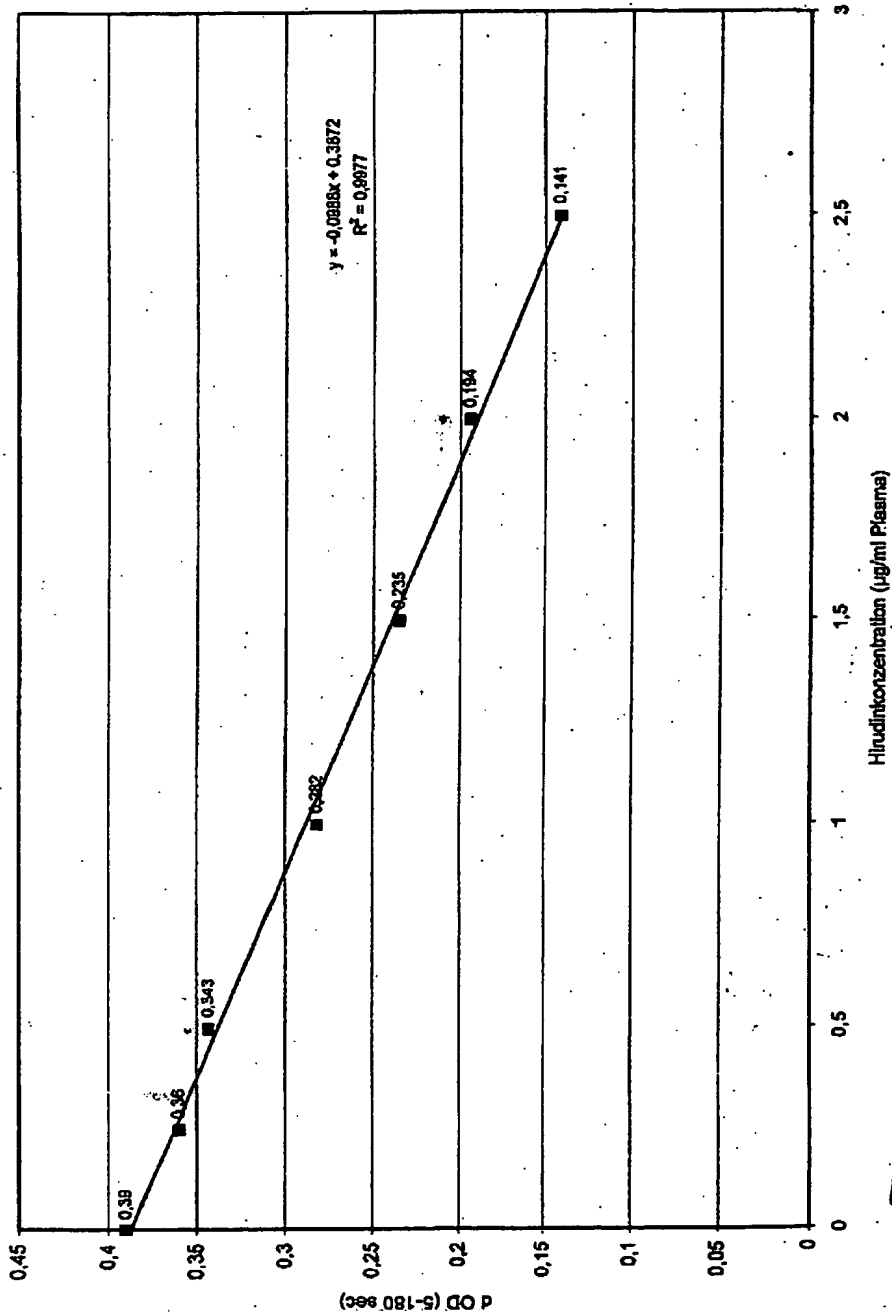


Fig. 1

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Method for determining the concentration
of thrombin inhibitors

5

Specification

10 The invention relates to a method for determining the
concentration of thrombin inhibitors, wherein body liquid is
taken from a living body and wherein a substance separating
prothrombin into meizothrombin or meizothrombin-des
fragment 1 (in the following Mtdesfgl) is added to said
body liquid. - As thrombin inhibitors are understood all
natural or synthetic substances directly inhibiting thrombin
or initial thrombin products. An example for a natural
15 thrombin inhibitor is hirudin, extracted from the saliva of
hirudo medicinalis. Hirudin is a very small protein com-
posed of 65 amino acids and having a molecular weight of 7
kD. Examples for synthetic thrombin inhibitors are the so-
called hirulogs comprising partial sequences being analo-
20 gous or homologous to hirudin, and polypeptides composed
of or comprising a tripeptide Phe-Pro-Arg or derivatives of
such a tripeptide, such as boric acid derivatives, chlo-
romethylketon derivatives, benzamidine derivatives, argini-
nals, amino acid modified derivatives and the like. The
25 above substances have probably the same mechanism effects
as hirudin. As donors of the body liquid are possible human
beings and mammals, such as rodents. Examples for body
liquids are in particular blood or blood plasma produced
from blood. But other body liquids not containing pro-
30 thrombin are also possible, for instance urine, liquor, sa-
liva, peritoneal liquid and others. Then, according to the
invention, prothrombin is added. Non-turbid means that
there should be no substantial amounts of suspended parti-
cles in the body liquid to be examined. This can be
35 achieved, if necessary, by centrifugation of the body liquid
and separation of the remainder.

The theoretical background the invention is based on is
the following. The transformation of prothrombin into

thrombin is an essential factor for blood coagulation. Thrombin acts on the creation of fibrin monomers from fibrinogen and on the polymerization of the fibrin monomers. Prothrombin is transformed into thrombin with the contribution of activated factor X, activated factor V Ca^{++} ions and phospholipids, such as platelet factor 3. A multi-step reaction takes place, with intermediates being formed in relatively small amounts. If however the coagulation is initiated by means of for instance ecarin or another snake venom or snake venom fraction, an "atypical" intermediate will be formed, such as meizothrombin, PIVKA meizothrombin or meizothrombin-des fragment 1 (PIVKA is the abbreviation for a protein being induced by a vitamin K antagonist). These atypical intermediates interestingly are inactivated for instance by hirudin, not however by heparin (factors IIa, IXa, XIa, XIIa inhibitor and/or antithrombin). Besides, they will also lead to thrombin formation and subsequently to coagulation. The affinity of hirudin and other synthetic thrombin inhibitors to the atypical intermediates is very high ($k_i > 10^{-10}$ mol/l for meizothrombin), so that the free atypical intermediate is temporarily bound by the thrombin inhibitor.

The above fundamentals are used in a method of the type referred to above, described in document US-A-5,547,850, wherein so to speak the consumption of the thrombin inhibitor is detected by measurement of the delay of coagulation. A large amount of thrombin inhibitor will lead to a long time before the beginning of coagulation, and vice versa. In principle, this method has proven successful in practical applications. The drawbacks however are that in cases of reduced fibrinogen level, falsifications may occur, since a (too) low fibrinogen level, same as a high thrombin inhibitor level, may lead to long coagulation times.

The invention is based on the technical object to specify a method for determining the concentration of thrombin inhibitors, said method providing precise values independently from the fibrinogen level.

For achieving this object, the invention teaches a method for determining the concentration of thrombin inhibitors in a non-turbid body liquid or a non-turbid extract from a body liquid, comprising the following steps: a) the
5 body liquid is taken from a living body, and the body liquid is subjected to a separation from the turbid matter, if necessary, b) to the non-turbid body liquid obtained in step a) are added a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or
10 Mtdesfgl, resp., a chromogenic or fluorogenic substrate not dissociable by active meizothrombin or Mtdesfgl, resp., and a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., and as an option prothrombin, c) the solution or mixture, resp., obtained in step b) is subjected to a
15 wavelength-selective light absorption or light emission measurement as a function of the time, d) from the reduction of the light absorption or light emission in step c) per time unit is determined the amount of the thrombin inhibitor included in the body liquid by comparison to previously
20 determined standard curves. Alternatively to the substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., or as a complement hereto, meizothrombin or Mtdesfgl, resp., may be added. Further, the invention teaches a method for determining the (specific) activity of
25 thrombin inhibitors (for inhibiting generated meizothrombin or Mtdesfgl, resp.) in a non-turbid aqueous liquid, comprising the following steps: a) a body liquid is taken from a living body, and the body liquid is subjected to a separation from the turbid matter, if necessary, or a non-turbid liquid
30 is synthetically produced, b) to the non-turbid body liquid obtained in step a) are added a given amount of thrombin inhibitor, if applicable a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., a chromogenic or fluoro-
35 genic substrate not dissociable by active meizothrombin or Mtdesfgl, resp., and a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., or meizothrombin or

Mt desfgl, resp., and as an option prothrombin, c) the solution or mixture, resp., obtained in step b) is subjected to a wavelength-selective light absorption or light emission measurement as a function of the time, d) from the reduction of the light absorption or light emission in step c) per time unit is determined the activity of the thrombin inhibitor by comparison (of the negative slope) to previously determined standard curves. - As chromogenic substrates are designated substances containing chromophoric groups and being specifically dissociated by thrombin, resulting in a coloration. Fluorogenic substances are substances that are specifically dissociated by thrombin, resulting in fluorescent substances. Prothrombin may be added, if the body liquid does not naturally contain sufficient prothrombin, for instance in the case of vitamin K deficiency, or if the amount of thrombin inhibitor to be expected or the activity of the thrombin inhibitor will recommend so, or if during an illness a prothrombin deficiency has occurred.

The invention is based on the surprising detection that chromogenic or fluorogenic substances being specifically dissociated by thrombin are equally specifically dissociable by meizothrombin or Mt desfgl, resp. This could not be expected since intermediates are necessary pre-steps, however do not naturally develop the same effects or reactivities as the thrombin. By that the detecting reaction according to the invention exclusively takes place by monitoring the meizothrombin or Mt desfgl inhibition, resp., by means of a color reaction, the detection is completely independent from the fibrinogen level. Rather, for body liquids, in particular blood or blood plasma, the coagulation has even to be prevented, in order to not disturb the color reaction evaluation. In addition, the determination of the concentration of the thrombin inhibitors is in all sections at least as accurate as the determination by means of the prior art method at a high fibrinogen level. Also, there is independence from any orally administered anti-coagulants possibly included in the liquid. Further advantages are: quick measurement within

minutes in chromogenic channels of conventional automatic coagulation devices (these often measure a turbidity at several wavelengths for the purpose of correction and therefore usually offer the possibility of the wavelength-selective and wavelength-variable light absorption measurement); high reproducibility of the found values because of very little variations of the individual values (the confidence interval is according to a multitude of test series below 5 %, usually 2.2 - 3.5 %); the high accuracy or reproducibility is further also achieved at very high thrombin inhibitor or hirudin levels, resp.; due to above features the method according to the invention is suitable for national and international standardization.

The method according to the invention is used on one hand in science, namely in all areas of examinations where concentrations of thrombin inhibitor have to be determined, and for the (if applicable, high-capacity) screening of prospective thrombin inhibitors. In the latter case, a multitude of synthetic prospective inhibitors can be examined with a high throughput with regard to their actual effects. Activity means here the determination whether at all an inhibition takes place, and if yes, how the kinetics or the specific activity are. On the other hand, clinical application is also a issue, for instance for monitoring the thrombin inhibitor levels of patients to whom the inhibitor is administered for therapeutical reasons. Thus it can be prevented, in a simple and economical way, that an under or over-dosage of the thrombin inhibitor takes place, and that in quasi-continuous or discontinuous monitoring.

In detail, the substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., may be selected from the group "calcium-complex forming agents, heparin, heparinoids, anti-thrombin III, protein C, fibrin polymerization inhibiting substances and mixtures of such substances". A specific example for this is Pefabloc FG manufactured by Pentapharm A; Bale, Switzerland, this substance being a tetrapeptide (Gly-Pro-Arg-Pro)

and preventing the fibrinogen polymerization with a high affinity. The substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., may be selected from the group of the snake venoms or snake venom fractions, for instance venoms of dispholidus, rhabdophis, bothrops, notechis, oxyuranus and Russel's vipers. Suitably cleaned fractions therefrom are used. Preferably, ecarin, a highly cleaned fraction of the echis-carinatus toxin, or multi-squamase, the prothrombin dissociating enzyme from echis multi-squamatus, is used. Such substances as for instance ecarin are commercially available from Pentapharm AG, Switzerland, among other sources.

The chromogenic substrate dissociable by active meizothrombin or Mtdesfgl, resp., may release p-nitroaniline under dissociation, and the light absorption measurement can then be performed at 405 nm. Examples for such or even other substrates are tripeptides available under the names Chromozym TH or Pefachrom TH from the companies Chromogenix, Boehringer, Pentapharm (Pefachrome TH is H-D-ChG-Ala-Arg-pN.2AcOH). An example for fluorochromic substrates is Pefachrom TH fluorogen, being available under the name Pefa 15865 from the company Pentapharm.

In detail, it is recommended for the activities in question to perform in step c) a first absorption or emission measurement after 0 - 100 s, preferably 0 - 50, most preferably 5 - 15 s, and a second one after another 10 - 1,000 s, preferably 50 - 500s, most preferably 150 - 300 s, measured from the addition of the substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp. The method according to the invention is particularly suited for the determination of hirudin or the determination of the concentration and/or the activity of synthetic thrombin inhibitors or hirulogs.

The invention also relates to a test kit for determining the concentration of thrombin inhibitors in a non-turbid body liquid or a non-turbid extract from a body liquid,

comprising the following kit components: K1) a solution of a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., K2) a chromogenic or fluorogenic substrate dissociable by active meizothrombin or Mtdesfgl, resp., and K3) a solution of a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., wherein component K3) may be replaced or complemented by a component K3a) of a solution with meizothrombin or Mtdesfgl, resp., and a test kit for determining the activity of thrombin inhibitors in a non-turbid body or in a non-turbid extract from a body liquid or in a non-turbid non-natural aqueous liquid, comprising the following kit components: as an option K1) a solution of a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., K2) a chromogenic or fluorogenic substrate dissociable by active meizothrombin or Mtdesfgl, resp., and K3) a solution of a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., wherein component K3) may be replaced or complemented by a component K3a) of a solution with meizothrombin or Mtdesfgl, resp. The kit components may be separated from each other or provided in a single test kit package. Further, as an optional additional kit component, a solution with prothrombin may be provided.

In any case it is understood that for the addition of substances dissociating thrombin, meizothrombin or Mtdesfgl, resp., and/or meizothrombin or Mtdesfgl, resp., these are used in defined, given amounts. Corresponding considerations apply to the substrate.

Based on the method according to the invention and being particularly well suited for screening purposes, further subject matter of the invention are thereby found or characterized new thrombin inhibitors, which are namely available by the following steps: A) elements of a group of prospective thrombin inhibitors are submitted subsequently or separately and simultaneously in a given and preferably

identical concentration to a method according to one of claims 2 to 8, B) the reduction of the light absorption or light emission per time unit is determined for each prospective thrombin inhibitor and compared to the light absorption or light emission per time unit of a given, preferably identical concentration of hirudin determined under identical conditions, C) those prospective thrombin inhibitors are selected the reduction of the light absorption or light emission of which per time unit corresponds to at least 10 % of the corresponding reduction when hirudin is used.

For the test kit according to the invention and the thrombin inhibitors found according to the invention apply the detailed explanations as given above for the method according to the invention.

As far as meizothrombin or Mtdesfgl, resp., is used, this can commercially be bought, for instance from Pentapharm AG, Switzerland, can however also be produced at immobilized ecarin according to the statement in document US-A-5,547,850.

The devices to be used for the invention are for instance semi or fully automatic coagulation devices being present anyway. These may for instance be automatic coagulation analyzers of the type Sysmex CA-500 or S2000 of the company Dade-Behring or of the type Electra 2000. In the CA-500, the light emitted by a LED is sent through a filter (405 nm) and then through the sample. The CA-500 determines in the chromogenic channel the variation or reduction of the light absorption of dyes, as for instance pNA (p-nitroaniline). If there is for instance hirudin in a sample, the generated or added meizothrombin or Mtdesfgl, resp., is inactivated, with the consequence of a thereby inhibited pNA release. The as such differently behaving (changing) optical density of the sample is recorded by a photodiode, and is evaluated. The monitored change in the light absorption is inversely proportional to the hirudin activity.

In the following, the invention will be explained in more detail, based on experiments representing examples of execution only.

For the determination of a standard curve, pooled human citrate plasma was treated with given amounts of hirudin solution. The thus obtained standard solutions were measured in a CA-500.

As reagents were filled in:

Reagent 1 [inhib] (room temperature): 400 µl Pefabloc FG (20 mM; dissolved in 0.9 % NaCl) + 2,100 µl Tris buffer;

Reagent 2 [chromo] (room temperature): Pefachrome TH (10 µmol/vial), diluted to 3 µmol/ml aq. dest,

Reagent 3 [ecarin] (15 °C): ecarin (50 EU/vial), diluted to .3 EU/ml (the contents of the ecarin bottle are dissolved in 5 ml of 0.9 % NaCl solution and shortly prior to application set to the final concentration with a 1:2 mixture of 0.9 % NaCl, containing 1 % Prionex (Merck) and 0.1 M CaCl₂ solution.

The test records are shown below. As dil. buffer was used a mixture of 16.6 µl prothrombin (cleaned; protein content 2.22 mg/ml) and 984 µl of a mixture of 900 µl Tris buffer (0.05 M, pH 8, 37 °C, + 0.1 M NaCl) and 100 µl Prionex (Merck).

Test records		
Name	Ecch	
Detector	Chrome	
Start Point	5 sec	
End Point	180 sec	
Sensitivity	Low Gain	
1 Sample Vol.	Citrate plasma	5 µl
Dil. Vol.	Buffer	70 µl
2 Sample Vol.	0 µl	
*****	0 µl	
Reagent 1	30 sec	
Reag. Vol.	Inhib	125 µl

<u>Rinse</u>		<u>125 μl</u>
Reagent 2		120 sec
Chromo	Chromo	20 μ l
<u>Rinse</u>		<u>100 μl</u>
Reagent 3		210 sec
Reag. Vol.	Ecarin	20 μ l
<u>Rinse</u>		<u>50 μl</u>

(Rinse: 1 % sodium hypochlorite solution)

Fig. 1 shows the obtained standard curve. The extremely good correlation coefficient of 0.9977 is conspicuous. In the experiment, just replace the standard sample by the sample to be determined, and read the unknown hirudin concentration in Fig. 1 from the measured reduction of the optical density.

PATENT CLAIMS

5 1. A method for determining the concentration of
thrombin inhibitors in a non-turbid body liquid or a non-
turbid extract from a body liquid, comprising the following
steps:

10 a) the body liquid is taken from a living body, and the body
liquid is subjected to a separation from the turbid matter, if
necessary,

15 b) to the non-turbid body liquid obtained in step a) are
added a coagulation-inhibiting substance not interfering in
the transformation prothrombin/active meizothrombin or
Mtdesfgl, resp., a chromogenic or fluorogenic substrate not
dissociable by active meizothrombin or Mtdesfgl, resp., and
a substance dissociating prothrombin into meizothrombin or
Mtdesfgl, resp., and as an option prothrombin,

20 c) the solution or mixture, resp., obtained in step b) is
subjected to a wavelength-selective light absorption or light
emission measurement as a function of the time,

25 d) from the reduction of the light absorption or light emis-
sion in step c) per time unit is determined the amount of the
thrombin inhibitor included in the body liquid by compari-
son to previously determined standard curves.

2. A method for determining the activity of thrombin
inhibitors in a non-turbid aqueous liquid, comprising the
following steps:

30 a) a body liquid is taken from a living body, and the body
liquid is subjected to a separation from the turbid matter, if
necessary, or a non-turbid liquid is synthetically produced,

b) to the non-turbid body liquid obtained in step a) are added a given amount of thrombin inhibitor, if applicable a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., a chromogenic or fluorogenic substrate not dissociable by active meizothrombin or Mtdesfgl, resp., and a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., or meizothrombin or Mtdesfgl, resp., and as an option prothrombin,

c) the solution or mixture, resp., obtained in step b) is subjected to a wavelength-selective light absorption or light emission measurement as a function of the time,

d) from the reduction of the light absorption or light emission in step c) per time unit is determined the activity of the thrombin inhibitor by comparison to previously determined standard curves.

3. A method according to claim 1 or 2, wherein the coagulation inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., is selected from the group "calcium-complex forming agents, heparin, heparinoids, anti-thrombin III, protein C, fibrin polymerization inhibiting substances and mixtures of such substances".

4. A method according to one of claims 1 to 3, wherein the substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., is selected from the group of the snake venoms or snake venom fractions.

5. A method according to one of claims 1 to 4, wherein the substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., is ecarin.

6. A method according to one of claims 1 to 5, wherein the chromogenic substrate dissociable by active meizothrombin or Mtdesfgl, resp., releases p-nitroanilin under dissociation, and the light absorption measurement is performed at 405 nm.

7. A method according to one of claims 1 to 6, wherein in step c) a first absorption or emission measurement after 0 - 100 s, preferably 0 - 50, most preferably 5 - 15 s, and a second one after another 10 - 1,000 s, preferably 50 - 500s, most preferably 150 - 300 s, measured from the addition of the substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., are performed.

8. A method according to one of claims 1 to 7, wherein the thrombin inhibitor is hirudin, a hirulog or a synthetic thrombin inhibitor.

9. A test kit for determining the concentration of thrombin inhibitors in a non-turbid body liquid or a non-turbid extract from a body liquid, comprising the following kit components: K1) a solution of a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., K2) a chromogenic or fluorogenic substrate dissociable by active meizothrombin or Mtdesfgl, resp., and K3) a solution of a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., wherein component K3) may be replaced or complemented by a component K3a) of a solution with meizothrombin or Mtdesfgl, resp.

10. A test kit for determining the activity of thrombin inhibitors in a non-turbid body or in a non-turbid extract from a body liquid or in a non-turbid non-natural aqueous liquid, comprising the following kit components: as an op-

tion K1) a solution of a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mt desfgl, resp., K2) a chromogenic or fluorogenic substrate dissociable by active meizothrombin or Mt desfgl, resp., and K3) a solution of a substance dissociating prothrombin into meizothrombin or Mt desfgl, resp., wherein component K3) may be replaced or complemented by a component K3a) of a solution with meizothrombin or Mt desfgl, resp.

11. A test kit according to claim 9 or 10, wherein the kit components are separated from each other but provided in a single test kit package.

12. A test kit according to claim 9 or 10, wherein as an optional additional kit component, a solution with prothrombin is provided.

13. Thrombin inhibitors, which are available by the following steps:

A) elements of a group of prospective thrombin inhibitors are submitted subsequently or separately and simultaneously in a given and preferably identical concentration to a method according to one of claims 2 to 8,

B) the reduction of the light absorption or light emission per time unit is determined for each prospective thrombin inhibitor and compared to the light absorption or light emission per time unit of a given, preferably identical concentration of hirudin determined under identical conditions,

C) those prospective thrombin inhibitors are selected the reduction of the light absorption or light emission of which per time unit corresponds to at least 10 % of the corresponding reduction when hirudin is used.

Abstract

The invention relates to a method for determining the concentration of thrombin inhibitors in a non-turbid body liquid or a non-turbid extract from a body liquid. It comprises the following steps. The body liquid is taken from a living body, and the body liquid is subjected to a separation from the turbid matter, if necessary. To the non-turbid body liquid thus obtained are added a coagulation-inhibiting substance not interfering in the transformation prothrombin/active meizothrombin or Mtdesfgl, resp., a chromogenic or fluorogenic substrate not dissociable by active meizothrombin or Mtdesfgl, resp., and a substance dissociating prothrombin into meizothrombin or Mtdesfgl, resp., and as an option prothrombin. The thus obtained solution or mixture, resp., is subjected to a wavelength-selective light absorption or light emission measurement as a function of the time. From the reduction of the light absorption or light emission per time unit is determined the amount of the thrombin inhibitor included in the body liquid by comparison to previously determined standard curves.

Fig. 1

Hirudin concentration ($\mu\text{g/ml}$ plasma)

0950554-110501

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR DETERMINING THE CONCENTRATION OF THROMBIN INHIBITORS

the specification of which

☐ is attached hereto

☒ was filed on 28 JANUARY 2000 as United States Application Number or PCT International Application Number PCT/DE00/00330 and (if applicable) was amended on _____

I hereby authorize our attorneys to insert the serial number assigned to this application.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 USC §119			
APPLICATION NO.	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
199 04 674.3	GERMANY	4 FEBRUARY 1999	YES

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

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APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under 35 U.S.C. §120 of any United States application, or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112.

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I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith: I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E. J. Branigan (20,565); John R. Moses (24,983); Harry B. Shubin (32,004); Brion P. Heaney (32,542); Richard J. Traverso (30,595); John A. Sopp (33,103); Richard M. Lebovitz (37,067); John H. Thomas (33,460); Catherine M. Joyce (40,668); Nancy J. Axelrod (44,014); James T. Moore (35,619); James E. Ruland (37,432); Jennifer J. Branigan (40,921) and Robert E. McCarthy (46,044)

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